



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵: C07C 47/58, 45/78, C12P 7/24	A1	(11) International Publication Number: WO 94/13614 (43) International Publication Date: 23 June 1994 (23.06.94)
(21) International Application Number: PCT/EP93/03481 (22) International Filing Date: 8 December 1993 (08.12.93) (30) Priority Data: 92311285.8 10 December 1992 (10.12.92) EP (34) Countries for which the regional or international application was filed: GB et al. (71) Applicant (for all designated States except US): QUEST INTERNATIONAL B.V. [NL/NL]; Huizerstraatweg 28, NL-1411 GP Naarden (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): BARRACLOUGH, Anthony, John [GB/GB]; Riverside, Mill Road, Wellingborough, Northampton NN8 1QW (GB). CHEETHAM, Peter, Samuel, James [GB/GB]; 28 Meadway, Harrold, Bedford MK43 7DR (GB). WESTCOTT, Roger, John [GB/GB]; 46 Castle Street, Wellingborough, Northampton NN8 1LW (GB). (74) Agent: DEKKER, Enno, E., J.; Unilever N.V., Patent Division, P.O. Box 137, NL-3130 AC Vlaardingén (NL).		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.
(54) Title: PRODUCTION OF VANILLIN		
(57) Abstract		
<p>A method of producing natural vanillin comprises reacting vanilla plant root material with ferulic acid or derivatives thereof (preferably ferulic acid derived from condensed molasses solubles) in the presence of adsorbent material (preferably charcoal) that releasably adsorbs vanillin but does not adsorb ferulic acid or derivatives thereof; and extracting vanillin from the adsorbent material, e.g. by solvent extraction. Ferulic acid is a precursor of vanillin, and the vanilla plant root material acts as a biocatalyst catalysing production of vanillin from ferulic acid (or derivatives thereof). The adsorbent material acts as a product reservoir for the vanillin produced, thus relieving possible product inhibition and/or further metabolism. The reaction also produces p-hydroxybenzaldehyde, at least some of which can be releasably adsorbed on the adsorbent material. The invention also provides a novel product comprising vanillin and p-hydroxybenzaldehyde at a vanillin: p-hydroxybenzaldehyde weight ratio of about 7.8:1.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

Production of Vanillin

Field of invention

This invention concerns the production of vanillin and
5 relates to a method of producing natural vanillin suitable
for use as vanilla flavour and the vanillin so produced.

Background to the invention

Vanilla/vanillin is very widely used as a flavour, for
10 instance in ice creams, a very wide range of foods, and
also in some fragrances. Vanilla beans contain about 2-3%
(w/w) vanillin (reference 1), and typically cost about
US\$70/lb. Vanilla bean extracts contain vanillin as a major
component but also contain a variety of other related
15 materials including p-hydroxy benzaldehyde (pOHB), 4-oxy-
benzyl alcohol, vanillyl alcohol, coumaric acid, 3,4-
dihydroxy-benzaldehyde, vanillin acid etc. (reference 5),
many of which contribute to the high organoleptic quality
of the extracts. Vanilla bean extracts currently vary in
20 price from about US\$ 100-300/Kg depending on quality,
especially vanillin content. The markets for these natural
products and for nature-identical synthetic material are
about US\$100M and US\$80M per annum, respectively, so that
although the natural vanilla market is only 13% of the
25 total by weight, it represents some 56% of the market by
value. This higher price is a reflection of the much higher
organoleptic value of bean-derived vanilla flavours. Thus
the vanillin present in vanilla beans can be calculated to
have a value of about US\$2,500/Kg.

30

Vanilla is a vine orchid that is very difficult to grow as
a commercial crop because of its exacting growth
requirements, and also because of the need to hand-
pollinate the flowers which are only fertile for one day.
35 Hence there is a significant need for alternative
commercial processes for producing natural vanilla or
vanillin, that are not subject to variations in supply,

quality and price.

Several approaches to the production of natural vanillin have been pursued. BASF have submitted a patent application
5 for the biotransformation of eugenol into vanillin using an Arthrobacter globiformis strain that does not further metabolise the vanillin via vanillic acid (reference 2). Similarly Serratia and Klebsiella strains have been used acting on eugenol and isoeugenol (reference 6) and
10 Paecilomyces variotti can produce vanillin from ferulic acid (reference 7).

Two approaches using plant cell technology have been reported: callus cultures (reference 8) and vanilla cells
15 selected for high production and secretion capability (reference 4). The pathway of vanillin synthesis, however, is not fully characterised yet (reference 3). Preliminary work showed that when phenylalanine was used as a precursor considerable amounts of p-coumaric acid accumulated,
20 indicating that its metabolism was a rate limiting step in the pathway and ferulic acid was the best precursor for further metabolism to vanillin.

Summary of the invention

25 In one aspect, the present invention provides a method of producing natural vanillin, comprising reacting vanilla plant root material with ferulic acid or derivatives thereof in the presence of adsorbent material that releasably adsorbs vanillin and extracting vanillin from
30 the adsorbent material.

Ferulic acid (3-methoxy-4-hydroxy-cinnamic acid) is a precursor of vanillin (4-hydroxy-3-methoxy-benzaldehyde), and the vanilla plant root material acts as a biocatalyst
35 catalysing production of vanillin from ferulic acid (or derivatives thereof). The adsorbent material acts as a product reservoir for the vanillin produced, thus relieving

possible product inhibition and/or further metabolism. The reaction also produces pOHB, at least some of which can be releasably adsorbed on to the adsorbent material.

- 5 The root material is conveniently in the form of small pieces, e.g. about 5mm lengths, although the form and size of the root material is not important. Indeed, it is possible to use whole plantlets. The root material may be obtained from plants grown conventionally, e.g. in a
10 glasshouse, or produced by tissue culture.

The adsorbent material may comprise a suitable ion exchange resin or other material. However, the currently preferred adsorbent material is charcoal, which is readily and
15 cheaply available and has suitable adsorbent properties.

The adsorbent material is preferably in finely divided or powdered form. The adsorbent material is desirably in close physical proximity to, and preferably in direct contact
20 with, the root material. The adsorbent material is preferably present at the start of the reaction, rather than being added later, as this is found to give better yields of vanillin.

- 25 The root material, ferulic acid (or derivatives) and adsorbent material are preferably incubated together under suitable conditions, e.g. in suitable growth medium containing sucrose, in an illuminated growth room at about 25°C under suitably sterile conditions with gentle
30 agitation, for a period of about 3 days. The adsorbent material is then removed and treated to extract the produced vanillin therefrom, while leaving unreacted ferulic acid (or derivatives) adsorbed thereto. The adsorbent material can be recycled for further use. The
35 root material can then be subjected to further treatment with ferulic acid (or derivatives) for a further 3 days with a new batch of adsorbent material (or the original

batch after vanillin removal). The process can be repeated several times, although the activity of the root material gradually declines with reuse, and in practice it is found that after at most 6 three day treatments vanillin
5 production has fallen to such a level that further treatment is not worthwhile. After the final 3 day treatment, vanillin is extracted from both the root material and the adsorbent material and the spent root material discarded. The vanillin extracted after the
10 various treatments can be pooled.

Vanillin is conveniently extracted from the adsorbent material (and also the root material) by mild alkaline hydrolysis, e.g. by treatment with 0.2 M KOH, followed by
15 selective solvent extraction, e.g. using diethyl ether. This extracts vanillin and some pOHB but not ferulic acid. Ether extraction was found to be very much more efficient than ethanol/water extraction, even when using specialist extraction equipment. The resulting extract may then be
20 dried to produce a product rich in natural vanillin and suitable for use as natural vanilla flavour.

The mild alkaline hydrolysis treatment has the effect of liberating the vanillin in soluble form. This indicates
25 that the vanillin produced by the method of the invention is substantially different to the vanillin glucoside formed in vanilla beans (reference 2) since virtually all the vanillin present as vanillin glucoside can be readily liberated by acid or beta-glucosidase treatment, whereas
30 acid hydrolysis treatment liberated only 20% of the vanillin produced by the root material. The conditions used were not, of course, sufficiently severe to release vanillin from lignified tissues.

35 Any unreacted ferulic acid (or derivative) remains adsorbed to the adsorbent material and can be recycled for further use. This allows for potentially complete conversion of the

ferulic acid. High conversion is economically important because the ferulic acid is a significant cost component in the overall process and because complete conversion of ferulic acid in one reaction can only be achieved using
5 very low concentrations of ferulic acid (less than 10ug/g dwt tissue) due to substrate and/or product initiation effects and due to the known toxicity of ferulic acid to plant tissues. Also the vanillin to pOHB ratio of the product produced at these very low ferulic acid
10 concentrations is significantly lower.

Ferulic acid or derivatives such as esters, e.g. ethyl ferulate, may be used in the method of the invention. However, ferulic acid itself is the favoured material as it
15 is most readily available.

Ferulic acid is desirably isolated from condensed molasses solubles (CMS), which represent an economically attractive source of natural ferulic acid. CMS contains about 125g
20 ferulic acid/tonne. Ferulic acid is conveniently isolated from CMS by treating CMS with industrial grade charcoal in finally divided or powdered form, either batch-wise or using charcoal packed into columns. The charcoal and extracted ferulic acid can then be used directly in the
25 method of the invention, with that charcoal constituting the absorbent material.

The treated CMS can then be recycled for use as an animal feed supplement, if desired, because the protein content
30 and total dissolved solids are essentially unaffected by removal of ferulic acid, so the product specification of CMS relevant to its use as an animal feed supplement is substantially unchanged. In this case, the real cost of the ferulic acid is equivalent to the cost of isolating it from
35 the CMS. Ferulic acid is also known to be present in very low concentrations in a number of beet molasses, but these represent an economically less attractive source of the

material.

The invention also includes within its scope vanillin produced by the method of the invention.

5

Using the method of the invention, vanillin productivities of about 400mg/Kg(dw) tissue/day and concentrations of about 7g Kg⁻¹ of root tissue can be regularly obtained. This concentration is about 35-fold greater than the
10 concentrations of vanillin originally present in the root tissue and is about 40% of that present in matured vanilla beans. Using roots supplied with ferulic acid, vanillin is produced 5-10 times faster than its normal synthesis in
15 precursor. The composition of the vanilla flavour produced using the method of the invention is comparatively close to that of vanilla beans; in particular it contains p-hydroxybenzaldehyde (the second most important component of vanilla flavour) at a vanillin: pOHB weight ratio of about
20 7.8:1, as compared to a ratio of 12.8:1 for bean derived vanilla. This may impart a superior organoleptic value and make the product of this root process more valuable.

In a further aspect, the invention also provides a product
25 comprising vanillin and p-hydroxybenzaldehyde at a vanillin : p-hydroxybenzaldehyde weight ratio of about 7.8:1.

The invention will be further described, by way of
30 illustration, in the following Example and by reference to the accompanying drawing in which:

Figure 1 is a schematic flow of a preferred method of the invention.

35

Example

Plant Material

Axenic tissue culture plantlets of Vanilla planifolia were maintained on hormone free Murashige and Skoog medium containing 2% sucrose and solidified with agar. Roots were harvested from such cultures aseptically, weighed and diced into approximately 5mm lengths. Some glasshouse grown roots were also used.

10

Reaction Mixture

25g of roots was added to 20g dry heat sterilised powdered charcoal (Darco G60 from Aldrich Chemicals) in Murashige and Skoog medium containing 2% sucrose and 500 μ moles of ferulic acid which was filter sterilised into the autoclaved medium. The mixture was incubated in an illuminated growth room at 25°C on an orbital shaker set at 100 rpm.

20 Harvest Extraction and Analysis

The medium and charcoal were decanted away from the roots which were washed to remove as much charcoal as possible. The roots were recycled with fresh medium and charcoal or extracted directly depending on the experiment. Filtered charcoal or washed roots were extracted as follows: Added to alkaline methanol (1M KOH/methanol 1:4) at a ratio of 1g wet weight to 5mls and heated at 80°C for 10 minutes. The alkaline methanol was recovered from the tissue/charcoal by centrifugation and the methanol evaporated at 80°C under vacuum. After cooling the extract was acidified to pH5 or less with 10M HCl before extraction with diethyl ether (20% of volume twice). The ethereal layers were collected, pooled and evaporated. The resultant material was taken up into water and aliquots analyzed for vanillin and p-hydroxy-benzaldehyde against authentic standards using reverse phase HPLC in known manner. Results are means of at least three replicates.

Using these techniques, a number of experiments were carried out and the following results were obtained.

RESULTS

- 5 Initial analysis showed that roots contain the highest concentration of vanillin compared to other somatic tissues (200 μ g/g DW). The addition of ferulic acid increases the vanillin concentration to 489 μ g/g after a 72 hour incubation. No increases could be seen after only 24 hours.
- 10 The ferulic acid concentration in the tissue increases twenty fold; however, the conversion rate is only 1.5% (Table 1).

- 80% of the vanillin produced from ferulic acid required
15 extraction with KOH in order to liberate it in a soluble form: this is substantially different to the vanillin glucoside formed in vanilla bean (reference 2) since virtually all the vanillin present as vanillin glucoside can be readily liberated by acid or beta-glucosidase
20 treatment, whereas acid treatment only liberated 20% of the vanillin produced by the aerial roots. Synthetic ethyl ferulate was also metabolised by the aerial roots.

- Considerable increases in the rate of synthesis of vanillin
25 and the concentrations of vanillin produced could be achieved by adding charcoal (Table 1).

- The results of a number of experiments are summarised in Table 1. In the column headed V, for rows 3, 4 and 5 three
30 figures are given: the top figure is root vanillin content, the middle figure is total vanillin content, and the bottom figure is charcoal vanillin content.

- The addition of ferulic acid increased the vanillin content
35 of the root tissue over a three day period. However, in the presence of charcoal, root vanillin content was reduced but overall vanillin production was higher when the charcoal

was extracted. Over a five day period with a change of charcoal after three days productivity was higher. The final charcoal sample which included that closely associated with the roots contained most vanillin.

5

In the presence of charcoal, vanillin and some other components as p-hydroxybenzaldehyde (pOHB) adsorb to the charcoal.

- 10 Charcoal has several beneficial effects. The inclusion of charcoal greatly increases the amounts of vanillin produced, and hence the volumetric productivity of the reactor, presumably by acting as a product "sink" and thereby relieving product inhibition and/or further
- 15 metabolism. The amounts of vanillin formed are selectively increased as compared to other metabolites such as p-hydroxybenzaldehyde; therefore the vanilla flavour produced has a composition closer to bean vanillin flavour than that produced in the absence of charcoal. The addition of
- 20 charcoal has little apparent stimulatory effect on p-hydroxybenzaldehyde production and most of the p-hydroxybenzaldehyde was found associated with the plant root tissue rather than the charcoal.
- 25 Some charcoal becomes closely physically associated with the root tissue and probably mediates precursor and product transfer into and out of the tissue, thus overcoming rate-limiting steps in the reaction. There is some evidence that much of the vanillin produced is present in this closely
- 30 associated charcoal. Certainly yields of vanillin were significantly reduced when the charcoal was maintained physically separate from the root tissue, and when the charcoal was added during the reaction rather than being present from the start. In addition, even after 1 day of a
- 35 3-day reaction substantial amounts of vanillin were already present in the charcoal.

In addition, reuse of the root tissue is facilitated by the use of charcoal. Greater yields of vanillin were obtained upon reuse of the root tissue, perhaps due to adaptation of the tissue, or more probably because of carry-over of some
5 of the closely associated charcoal that contains much of the vanillin produced. In these experiments some of the root tip material continued to grow, but this new plant material probably does not make a significant additional contribution to the ferulic acid transforming activity of
10 the aerial roots.

The aerial root tissue was reused four times over a two week period. Rates of vanillin formation of 400mg.Kg^{-1} dw/day were obtained which is about 5-fold greater than
15 occurs in vanilla beans; and a concentration of 7g vanillin Kg^{-1} dw tissue obtained which is about 40% of that present in vanilla beans. These rates and productivities are also far in excess of the corresponding values quoted for plant cell culture or plant callus tissue culture techniques
20 (references 4 and 10). However, only low intracellular concentrations of ferulic acid could be detected indicating that its transfer into the root tissue is rate-limiting.

In addition although the original medium contained 2.0g/l
25 sucrose, no reduction in vanillin production occurred when this sucrose was reduced to 0.1g/l so as to reduce media costs.

The size of the root pieces used had little effect on
30 activity; indeed, initial experiments show that whole plantlets can be used for the conversion of ferulic acid into vanillin.

The material produced by the roots in the presence of
35 charcoal contained concentrations of p-hydroxybenzaldehyde in a ratio to the vanillin present that is quite close (7.8 vanillin: 1 pOHB) to that present in vanilla bean extracts

(12.8:1). The other minor components of vanilla such as vanillic acid are not present. Some residual ferulic acid remains, but this can be removed by treatment with suitable resins such as Amberlite A26 or 27.

5

Most of the vanillin formed and some of the p-hydroxy-benzaldehyde partitions into the charcoal from which it can be easily extracted in a purer and more concentrated form. Extraction with diethyl ether is preferred because it
10 selectively extracts the vanillin, but not the remaining ferulic acid. Thus the charcoal and remaining ferulic acid can be recycled and reacted. This feature of the process allows potentially complete conversion of the ferulic acid.

15 As mentioned above, the ferulic acid is desirably derived from condensed molasses solubles (CMS), which are available commercially, e.g. from Distillers. A preferred production route for producing vanillin by the method of the invention, based on extraction of ferulic acid from CMS, is
20 illustrated schematically in Figure 1.

References

1. Clark, G.S. Perfumer and Flavourist 15 (1990), 45-54
25
2. Cooper, B. DE 3604874A1
3. Funk, C. and Brodelius, P. Phytochem 29 (1990), 845-848
30
4. Knuth, M.E. and Sahai, O.P. (1989) WO 89/00820
5. Leong, G., Uzio, R. and Desbesy, M. Flavour and Fragrance J. 4 (1989), 163-167.
35
6. Rabenhorst, J. and Hopp, R. EP 0405197A1

7. Rahouti, M., Seigle-Murandi, F., Steiman, R. and Eriksson, K.E. Appl. and Envir. Microb. 55 (1989), 2391-2398.
- 5 8. Romognoli, L.G. and Knorr, D. Food Biotechnology, 2 (1988), 93-104.

TABLE 1

10 Total vanillin (V), ferulic acid (FA) and p-hydroxybenzaldehyde (p.OHB) content of vanilla tissues and charcoal after alkaline hydrolysis ($\mu\text{g/gDW}$ of tissue)

15

		FA	V	p.OHB	$\frac{V}{p.OHB}$	
	1	Vanilla Roots	257	165	539	0.31
	2	Vanilla Roots + 0.5mM FA (3 days)	1995	541	529	1.02
	3	Vanilla Roots + 0.5mM FA (3 days) + charcoal		33 (1101) 1068	673	1.63
	4	As (3) (2 x 3 days) + charcoal		27 (3675) 3648	979	3.75
20	5	As 3 (5 x 3 days) + charcoal		40 (7100) 7060	910	7.80
	6	Vanilla beans (dry)		15000	1170	12.81

CLAIMS

1. A method of producing natural vanillin, comprising
reacting vanilla plant root material with ferulic acid
or derivatives thereof in the presence of adsorbent
material that releasably adsorbs vanillin; and
extracting vanillin from the adsorbent material.
2. A method according to claim 1, wherein the adsorbent
material is charcoal.
3. A method according to claim 1 or 2, wherein the
adsorbent material is in finely divided or powdered
form and is in direct contact with the root material.
4. A method according to claim 1, 2 or 3, wherein the
adsorbent material is present at the start of the
reaction.
5. A method according to anyone of the preceding claims,
wherein the root material, ferulic acid (or
derivatives) and adsorbent material are incubated
together under suitable conditions for a period of
about 3 days; the adsorbent material is then removed
and treated to extract vanillin therefrom, while
leaving unreacted ferulic acid (or derivatives)
adsorbed thereto; and the root material is subjected
to one or more further similar treatments with ferulic
acid (or derivatives) for a further period of about 3
days with a new batch of adsorbent material followed
by removal of the adsorbent material, and extraction
of vanillin therefrom, with vanillin also being
extracted from the root material after the final
treatment.
6. A method according to claim 5, wherein the root
material is subjected to a total of up to 6 treatments

each for a period of about 3 days.

7. A method according to any one of the preceding claims,
wherein vanillin is extracted from the adsorbent
5 material and the root material by mild alkaline
hydrolysis followed by solvent extraction.
8. A method according to claim 7, wherein the solvent
extraction is performed using diethyl ether.
- 10 9. A method according to any one of the preceding claims,
wherein the adsorbent material is recycled for further
use in the method of the invention after extraction of
vanillin therefrom.
- 15 10. A method according to any one of the preceding claims,
using ferulic acid.
- 20 11. A method according to claim 10, wherein the ferulic
acid has been isolated from condensed molasses
solubles (CMS) by treatment with charcoal.
- 25 12. A method according to claim 11, wherein the charcoal
and extracted ferulic acid are used directly in the
method of the invention with the charcoal constituting
the adsorbent material.
- 30 13. A method according to claim 11 or 12, wherein the
treated CMS is recycled for use as an animal feed
supplement.
14. A method of producing natural vanillin, substantially
as herein described.
- 35 15. Vanillin produced by the method of any one of claims 1
to 14.

16. A product comprising vanillin and p-hydroxybenzaldehyde at a vanillin: p-hydroxybenzaldehyde weight ratio about 7.8:1.

1/1

Fig.1.

PROPOSED PROCESS FOR PRODUCING VANILLA USING AERIAL ROOTSRAW MATERIALS

VANILLA ROOTS

CONDENSED
MOLASSE SOLUBLES (CMS)EXTRACTION OF FERULIC
ACID WITH CHARCOALTREATED CMS FOR ANIMAL
FEED (SIDE-PRODUCT)

FERULIC ACID ON CHARCOAL

REACTIONREACTION CONTAINING ROOTS,
FERULIC ACID AND CHARCOALRECYCLE AND REUSE
OF ROOT TISSUESELECTIVE EXTRACTION
OF CHARCOALPRODUCT ISOLATION
AND PURIFICATIONRECYCLE UNUSED
FERULIC
ACID ON CHARCOAL

DRY CHARCOAL EXTRACT

NATURAL VANILLA FLAVOUR

INTERNATIONAL SEARCH REPORT

Intern: Application No
T/EP 93/03481

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07C47/58 C07C45/78 C12P7/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12P C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,890 082 (ESCA GENETICS CORPORATION) 9 February 1989 cited in the application see the whole document ---	1,2
A	US,A,5 128 253 (I.M. LABUDA ET AL.) 7 July 1992 see the whole document -----	1

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

17 February 1994

Date of mailing of the international search report

25.02.94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Bonnevalle, E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 93/03481

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-890082		NONE	
US-A-5128253	07-07-92	US-A- 5279950	18-01-94

Form PCT/ISA/210 (patent family annex) (July 1992)